ELSEVIER

Molecular and Cellular Probes 19 (2005) 213–217

Molecular and Cellular Probes

www.elsevier.com/locate/ymcpr

Rapid PCR detection of enterohemorrhagic *Escherichia coli* (EHEC) in bovine food products and feces

Jay L.E. Ellingson[a,*], Jeff J. Koziczkowski[a], Jennifer L. Anderson[a], Steve A. Carlson[b], Vijay K. Sharma[b]

[a]*Food Safety Services, Division of Laboratory Medicine, Marshfield Clinic Laboratories-Food Safety Services, 1000 North Oak Avenue, Marshfield, WI 54449, USA*

[b]*Pre-harvest Food Safety and Enteric Diseases Research Unit, United States Department of Agriculture, Agricultural Research Center, National Animal Disease Center, Ames, IA 50010, USA*

Received 29 October 2004; accepted for publication 12 January 2005

Abstract

Although *Escherichia coli* (*E. coli*) O157:H7 is a major cause of foodborne illness, other types of *E. coli* can also cause illness. *E. coli* that possess the *eae* gene for attachment and effacing have the potential to cause disease. Many real-time, molecular-based assays have been developed to detect Enterohemorrhagic *E. coli* (EHEC) including *E. coli* O157:H7. However, no assay currently exists to detect the *eae* gene present in *E. coli* O157:H7 and other EHEC strains with a confirmed positive or negative result in less than 12 h.

Raw beef food products (raw ground beef and raw boneless beef) at 25 and 375 g samples and bovine fecal samples at 2 g were inoculated with 10^1, 10^3, 10^4, and 10^5 organisms of *E. coli* O157:H7 to test the sensitivity of this assay. Fourteen different foodborne bacteria (including *E. coli* O157:H7) and 19 various *E. coli* strains, obtained from the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) were tested for specificity. *E. coli* O157:H7 was detected at the level of 10^1 organisms in both 25 and 375 g samples of raw ground and raw boneless beef products as well as 2 g samples of bovine feces after pre-enrichment and concentration. None of the 14 foodborne bacteria screened for cross-reactivity was detected. All USDA *E. coli* strains confirmed to contain the *eae* gene were detected.

Keywords: PCR; Enterohemorrhagic *Escherichia coli*; *eae* gene; Raw beef products; Bovine feces

1. Introduction

Escherichia coli (*E. coli*) 0157:H7 is a major cause of foodborne illness. An estimated 73,000 cases of infection and 61 deaths occur annually in the United States, due to this bacterium (CDC). *E. coli* O157:H7 infections cause hemorrhagic colitis and are associated with hemolytic uremic syndrome [1,2]. Cattle are a natural reservoir for *E. coli* O157:H7. Every year, many people in the US [3] and developing countries [4] are infected from consuming undercooked beef. While *E. coli* O157:H7 infections have had wide publicity, other types of *E. coli* can cause similar illness, with symptoms such as bloody diarrhea, abdominal cramps, and fever. Recent publications have shown that these other *E. coli* strains are often found in food products [5–7].

The broad group of *E. coli* known as Enterohemorrhagic *Escherichia coli* (EHEC), including *E. coli* O157:H7, contain the *eae* gene for attachment and effacing. *E. coli* possessing this gene have the potential to attach to the intestinal lining and cause foodborne illness [7,8]. *E. coli* O157:H7 is a pathogen regulated by the United States Department of Agriculture (USDA) and Food and Drug Administration (FDA), therefore, food-manufacturing plants are subject to having their products tested for *E. coli* O157:H7 contamination. However, testing for the presence of other disease causing *E. coli*, such as O111 and O26 strains, are not currently required.

Many molecular-based *E. coli* assays have been developed. Several polymerase chain reaction (PCR) [9–11], reverse transcriptase PCR (RT-PCR) [12,13], and real-time PCR [14,15] tests have been developed to detect *E. coli*

* Corresponding author. Tel.: +1 715 389 3430; fax: +1 715 389 7599.
E-mail address: ellingson.jay@marshfieldclinic.org (J.L.E. Ellingson).

0890-8508/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.mcp.2005.01.004

O157:H7. However, no test used currently detects the *eae* gene and make it possible to obtain confirmation of negative or positive test results in less than 12 h from the receipt of a sample.

This article describes the development of a PCR based assay for *E. coli*, which targets the *eae* gene for attachment and effacing.

2. Materials and methods

2.1. Sample preparation

Twenty-five and 375 g samples of each raw ground beef, raw boneless beef, processed beef, and two grams of bovine fecal material were weighed into sterile whirlpak filter bags. Each sample was diluted with modified Tryptic Soy Broth (1:10; weight to volume). Novobiocin was added to each sample to a final concentration of 0.002%. Spectrophotometer absorbance of a log phase *E. coli* O157:H7 culture was measured at 600 nm. The absorbance was used to calculate the original concentration of *E. coli* O157:H7 and the desired dilution levels. All inoculation levels were verified by plating each dilution on standard methods agar, incubating 2 days at 35–37 °C, and enumerating the colonies on each plate. Raw beef food products (25 and 375 g samples) and bovine fecal samples were inoculated with 10^1, 10^3, 10^4, and 10^5 organisms of *E. coli* O157:H7 to test for sensitivity. One negative (not spiked with *E. coli* O157:H7) raw beef food product (25 and 375 g samples) or bovine fecal sample was included with each batch to monitor for any initial product contamination. The samples were mechanically homogenized for 50 s and then incubated at 35–37 °C for 6 h in a shaker incubator set at 100 rpm. An aliquot of each sample (15, 50, or 2 ml from 25, 375, and 2 g samples, respectively) was then centrifuged at 2500*g* for 10 min. The supernatant was discarded and the remaining pelleted material was resuspended in 200 μl TE buffer (1 M; pH 7.5) and extracted using the QIAamp DNA mini kit (QIAGEN, Valencia, CA) according to the manufacturers' instructions.

Fourteen different foodborne bacteria (including *E. coli* O157:H7) and 19 various *E. coli* strains were obtained from the United States Department of Agriculture-Agricultural Research Service (USDA-ARS). These bacteria were also grown up in modified Tryptic Soy Broth and extracted using the QIAamp DNA mini kit. The samples were then screened in duplicate to determine inclusivity and exclusivity of this testing method.

2.2. PCR amplification

Oligonucleotide primers were designed to provide for the PCR amplification of a 360 bp product spanning from base 1179 to 1539 of the *eae* gene of the EHEC genome (GenBank accession no. AF081182). In addition, internal hybridization probes were designed to allow for detection of the PCR product by Fluorescence Resonance Energy Transfer (FRET) within the LightCycler (Roche). These probes anneal to the upper strand from positions 1477–1495 (upstream) and 1497–1516 (downstream). The hybridization probes are sequence specific and designed to only bind to the target DNA sequence. During the annealing step of PCR, the probes hybridize to the targeted DNA sequence. Then the fluorochrome on the 3′ end of the first probe is excited by an external light source. The close proximity (1 bp) of this first fluorochrome to a second fluorochrome on 5′ end of a second probe allows for the transfer of energy to take place. The second probe then emits a measurable amount of light. This measurable amount of light is proportional to the amount of product made, therefore, the progress of the reactions can be monitored during each run. The amplified product can then be slowly heated (0.1°/s), while the fluorescence is monitored. The fluorescence of the product will decrease when one of the two probes melts off of the product. This melting temperature, T_m, is specific to the targeted product. The sequences of the primers and fluorescently labeled hybridization probes are presented in Table 1.

Table 1
Oligonucleotide primers and fluorescently labeled hybridization probes specific for the *E. coli eae* gene

Primer	Sequence
Forward primer	TGGTACGGGTAATGAAAA
Reverse primer	AATAGCCTGGTAGTCTTGT
Upstream probe	CGCAGTCAGGGCGGTCAGA-FLUORESCEIN
Downstream probe	LC RED 640-TCAGCATAGCGGAAGCCAAA-PHOSPHORYLATION

The LightCycler DNA Master Hybridization Probes kit (Roche Diagnostics, Indianapolis, IN) was used according to the manufacturers' instructions. The amplification mixture contained the following concentrations of reactants 1 μM each primer, 0.2 μM each probe, and 3 mM MgCl. Each set of samples included a negative control (in which pcr-grade water was substituted for a sample). The amplification reactions containing 17 μl of master mix and 3 μl extracted DNA template was added to each capillary. Amplification conditions were as follows: 2 min at 95 °C; 55 cycles of 0 s at 95 °C, 5 s at 53 °C with a single fluorescence acquisition during each cycle; and 10 s at 72 °C. Melting curve conditions are as follows: 0 s at 95 °C; 15 s at 35 °C; and 0 s at 85 °C with a 0.1 C/s slope and continuous acquisition. Cooling cycle was 30 s at 40 °C.

3. Results

All 25 and 375 g samples of raw ground beef, raw boneless beef, and processed beef and the two grams samples of bovine fecal material showed amplification and detection down to the level of 10^1 *E. coli* O157:H7

Table 2
PCR amplification and melting curve analysis results for raw ground beef, raw boneless beef, and bovine fecal material spiked with *E. coli* O157:H7

Sample	Inoculum	PCR		Melting curve analysis	
		25 g	375 g	25 g	375 g
Raw ground beef	10^1	+	+	+	+
	10^3	+	+	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+
		25 g	375 g	25 g	375 g
Raw boneless beef	10^1	+	+	+	+
	10^3	+	+	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+
		2 g		2 g	
Bovine fecal material	10^1	+		+	
	10^3	+		+	
	10^4	+		+	
	10^5	+		+	

All samples were tested in duplicate and all yielded the same result in both tests.

organisms prior to pre-enrichment and concentration (Table 2). Fig. 1a shows the amplification of the *eae* gene of different concentrations of *E. coli* O157:H7 inoculated into 375 g samples of raw ground beef. The melting of the hybridization probes off of the amplified product produces a drop in fluorescence, when graphed as the inverse, produce a distinctive peak at a predictable temperature, confirming the product of interest (Fig. 1b).

None of the 14 foodborne bacteria screened for cross-reactivity showed amplification except the positive control,

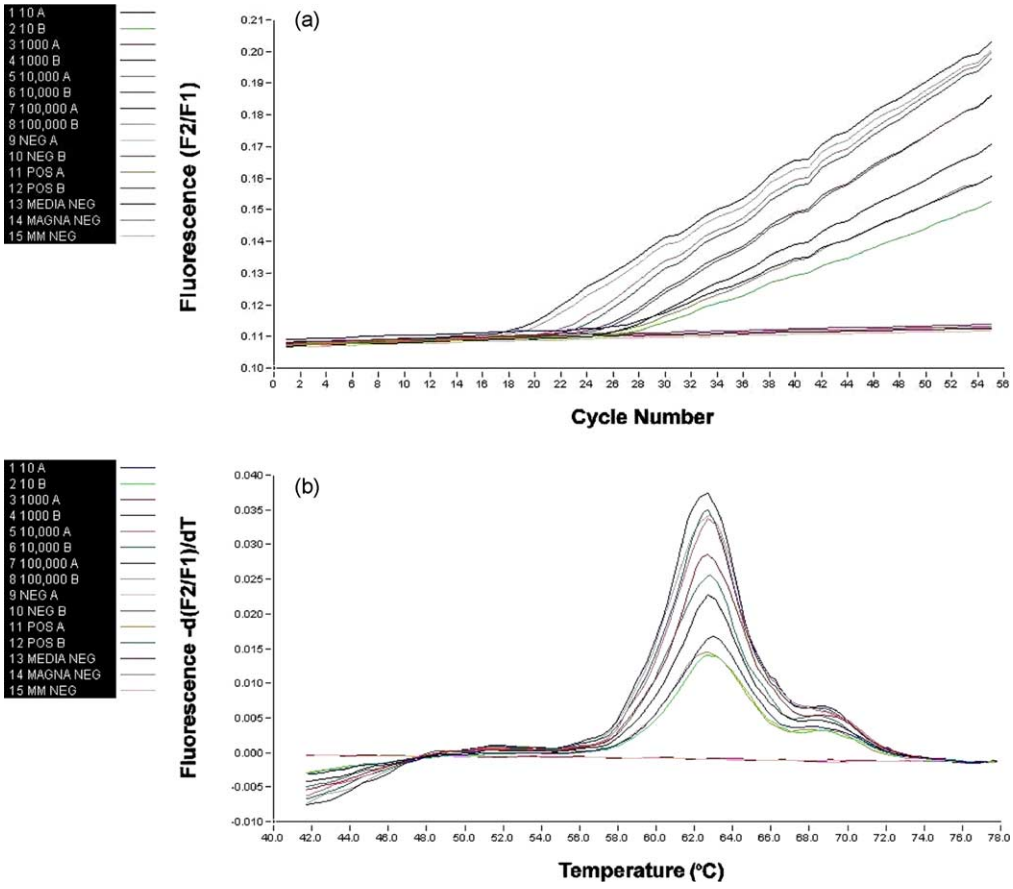

Fig. 1. The amplification (a) and melting curves (b) of the *eae* gene from different concentrations of *E. coli* O157:H7 inoculated into 375 g samples of raw ground beef. Concentrations as low as 10^1 organisms of *E. coli* O157:H7 are detectable in 375 g of raw ground beef. All samples were tested in duplicate.

Table 3
E. coli eae gene PCR inclusivity and exclusivity results

Sample ID		PCR result	Melting curve analysis
Shigella flexneri		−	−
Listeria fermentum		−	−
Pantoa agglomerans		−	−
Enterobacter faecalis		−	−
Citrobacter freundii		−	−
Staphlococcus aureus COAG+		−	−
Salmonella abaetetuba		−	−
Escherichia coli O157:H7		+	+
Bacillus cereus		−	−
Listeria monocytogenes		−	−
Klebsiella pneumoniae		−	−
Escherichia. coli—generic		−	−
Listeria innocua		−	−
Escherichia vulneris		−	−
USDA Escherichia coli isolates			
MRU-04	O145:NM	+	+
MRU-11	O145:NM	+	+
MRU-26	O145:NM	+	+
MRU-15	O157:H7	+	+
MRU-16	O157:H7	+	+
MRU-18	O157:H7	+	+
MRU-19	O157:H7	+	+
MRU-20	O157:H7	+	+
MRU-21	O157:H7	+	+
MRU-12	O157:NM	+	+
MRU-13	O157: other than H7	−	−
MRU-27	O157: other than H7	−	−
MRU-28	O157: other than H7	−	−
MRU-29	O157: other than H7	−	−
MRU-30	O55:H7	+	+
MRU-31	O55:H7	+	+
MRU-32	O55:H7	+	+
MRU-33	O55:H7	+	+
MRU-17	O55:H7	+	+

All samples were tested in duplicate and all yielded the same result in both tests.

E. coli O157:H7 isolate (Table 3). Fifteen of the 19 EHEC strains from the USDA were detected with this test (Table 3).

4. Discussion

The use of this *E. coli eae* gene detection methodology is highly sensitive, detecting as low as 10^1 organisms in 375 g of raw or processed beef.

Nineteen *E. coli* strains were obtained from the USDA for testing. After testing was completed, the USDA revealed to us that four O157: other than H7 isolates obtained from them were known not to contain the *eae* gene, but all others did contain it. All 15 isolates containing the *eae* gene obtained from the USDA tested in this study were detected. The assay proved highly specific at detecting only *E. coli* strains containing the *eae* attachment and effacing gene. Although the *sip* B-*sip* C genes in *Salmonella* and the attachment and invasion genes in *Shigella* contain homologies to the *eae* gene in *E. coli* O157:H7 [16–18], the *Salmonella* and *Shigella* isolates tested do not cross-react with this test.

The use of hybridization probes in combination with subsequent melting curve data aids in product confirmation and could allow other primer and hybridization probe sets to be added to this assay to detect shiga-toxin producing *E. coli* genes (STEC) or other *E. coli* genes of interest. These other hybridization probes could be designed to have a different melting temperature or use a different fluorescent label than that of the *eae* gene hybridization probes.

The technique developed here for the quantitative, real-time PCR detection of EHEC dramatically reduces the reporting time compared to standard EIA, Diagnostic Culture, and other PCR methods. The method described can be used to quantify the number of genomic equivalents in each reaction with the use of standards. This assay could be used by food manufacturing plants in the future to track origins of contamination with quantification. There may even be allowable limits of contamination in some sectors of the food industry and tools like this would enable minimally contaminated lots to still be sold for certain uses.

The ability to provide *E. coli* O157:H7 test results in 12 h or less is of great benefit to food companies and the consumers of their products. Providing confirmed *E. coli* O157:H7 test results more rapidly should encourage and enable food companies to test more of their production prior to its distribution. This should reduce the amount of food products contaminated with *E. coli* O157:H7 in commerce thus reducing foodborne illness cases caused by it. More rapid turn-around time for test results will also reduce inventory and warehousing costs for food companies and provide fresher products to consumers.

5. Conclusions

The *E. coli eae* gene PCR assay described in this paper is sensitive down to 10^1 *E. coli* O157:H7 organisms in a 375 g sample. This assay can both detect and confirm the presence or absence of *E. coli* O157:H7 with great sensitivity and provide a result within 12 h upon receipt of sample. This test methodology can also detect other *E. coli* isolates containing the *eae* gene, that can cause disease, such as 0111 and 026 strains or can be paired with an Immunomagnetic Separation (IMS) system to detect only *E. coli* O157:H7 in less than 12 h.

Acknowledgements

We would like to thank Dr Mohammed Koohmaraie and his staff at the USDA-ARS for providing EHEC isolates for this study.

References

[1] Griffin PM, Tauxe RV. The epidemiology of infections caused by *Escherichia coli* 0157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol Rev 1991;13: 60–98.

[2] Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, et al. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 1983;308:681–5.

[3] Bell BP, Goloft M, Griffin PM, Davis MA, Gordon DC, Tarr PI, et al. A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. The Washington experience. JAMA 1994;17:1349–53.

[4] Huilan S, Zhen LG, Mathan MM, Mathew MM, Olarte J, Espejo R, et al. Etiology of acute diarrhoea among children in developing countries: a multicentre study in five countries. Bull World Health Organ 1991;69:549–55.

[5] Drysdale M, MacRae M, Strachan NJ, Reid TM, Ogden ID. The detection of non-O157 *E. coli* in food by immunomagnetic separation. J Appl Microbiol 2004;97:220–4.

[6] Gonzales, Garcia EA. Animal health and foodborne pathogens: enterohemorrhagic O157:H7 and other pathogenic *Escherichia coli* virotypes (EPEC, ETEC, EIEC, EHEC). Pol J Vet Sci 2002;5: 103–15.

[7] Sharma VK. Detection and quantitation of enterohemmorrhagic *Escherichia coli* O157, O111, and O26 in beef and bovine feces by real-time polymerase chain reaction. J Food Prot 2002;65:1371–80.

[8] Louie M, Azavedo JC, Handelsman MY, Clark CG, Ally B, Dytoc M, et al. Expression and characterization of the eaeA gene product of *Escherichia coli* serotype O157:H7. Infect Immun 1993;61:4085–92.

[9] Bayardelle P, Zafarullah M. Development of oligonucleotide primers for the specific PCR-based detection of the most frequent Enterobacteriaceae species DNA using wec gene templates. Can J Microbiol 2002;2:113–22.

[10] Osek J. Rapid and specific identification of shiga toxin-producing *Escherichia coli* in faeces by multiplex PCR. Lett Appl Microbiol 2002;34:304–10.

[11] Campbell GR, Prosser J, Glover A, Killham K. Detection of *Escherichia coli* O157:H7 in soil and water using multiplex PCR. J Appl Microbiol 2001;91:1004–10.

[12] Yaron S, Matthews KR. A reverse transcriptase-polymerase chain reaction assay for detection of viable *Escherichia coli* O157:H7: investigation of specific target genes. J Appl Microbiol 2002;4: 633–40.

[13] McIngvale SC, Elhanafi D, Drake MA. Optimization of reverse transcriptase PCR to detect viable Shiga-toxin-producing *Escherichia coli*. Appl Environ Microbiol 2002;68:799–806.

[14] Jothikumar N, Griffiths MW. Rapid detection of *Escherichia coli* O157:H7 with multiplex real-time PCR assays. Appl Environ Microbiol 2002;6:3169–71.

[15] Belanger SD, Boissinot M, Menard C, Picard FJ, Bergeron MG. Rapid detection of Shiga toxin-producing bacteria in feces by multiplex PCR with molecular beacons on the smart cycler. J Clin Microbiol 2002;40: 1436–40.

[16] Galan JE. Molecular and cellular bases of *Salmonella* entry into nonphagocytic cells. Curr Top Microbiol Immunol 1995;209: 43–60.

[17] Donnenberg MS, Yu J, Kaper JB. A second chromosomal gene necessary for intimate attachment of enterpathogenic *Escherichia coli* to epithelial cells. J Bacteriol 1993;175:4670–80.

[18] Ellingson JL, Anderson JL, Carlson SA, Sharma VK. Twelve hour real-time PCR technique for the sensitive and specific detection of *Salmonella* in raw and ready-to-eat meat products. Mol Cell Probes 2004;18:51–7.